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Mucosal and systemic immunogenicity of a recombinant, non-ADP-ribosylating pertussis toxin: effects of formaldehyde treatment

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The effect of formaldehyde treatment on the mucosal and systemic immunogenicity of the genetically detoxified pertussis toxin (PT-9K/129G) was investigated. Groups of BALB/c were immunized intranasally (i.n.) or subcutaneously (s.c.) with untreated, lightly formaldehyde treated (LFT) or heavily formaldehyde treated (HFT) recombinant pertussis toxin (PT) mutant, PT-9K/129G. Intranasal immunization with native PT-9K/129G induced significant levels of anti-toxin antibodies in serum and IgA anti-toxin responses in nasal and lung lavages of these mice. Similar local and systemic responses were observed following intransal immunization with LFT toxin. However, i.n. immunization with HFT toxin failed to induce a local IgA response and elicited a much diminished anti-toxin response in the serum. In contrast, the total antibody response following s.c. immunization was not significantly affected. In addition, i.n. immunization with native PT-9K/129G induced low but detectable levels of toxin neutralizing antibodies in the serum. These results show that native PT-9K/129G protein acts as a mucosal immunogen in mice and that this activity is greatly diminished by HFT of the protein.

Keywords: Pertussis toxin; mucosal immunization; formaldehyde

Many pathogens initially colonize the host at or through a mucosal surface[1,2]. To defend against such pathogens and their products, both the innate and antigen specific mucosal immune systems are activated[3,4]. Direct presentation of antigen to the mucosal surface is usually necessary to generate a local mucosal and may also induce a systemic response. However, in general delivery of soluble non-living antigens to mucosal surfaces stimulates poor or no protective immunity which is one reason why most vaccines are given parenterally[5].

There are only a few well documented examples of defined, non-living soluble antigens that can stimulate an immune response when administered mucosally in small (microgram) quantities. Perhaps the best known examples are cholera toxin (CT), the related *Escherichia coli* Heat-Labile Toxin (LT) and their respective B subunits (CT-B and LT-B)[6,7]. Both these holotoxins and their B subunits alone, generate strong local and systemic anti-toxin antibody responses following oral or intranasal (i.n.) immunization[8–10].

Bordetella pertussis, the causative agent of whooping cough, is a mucosal pathogen in which infection is restricted to the mucosa of the respiratory tract[11]. Pertussis toxin (PT) is perhaps the best characterized virulence factor and immunogen of *B. pertussis*. Like CT, it is a bipartite toxin composed of enzymatic and binding subunits. A single polypeptide, S1, specifies the ADP-ribosyltransferase activity, whilst the eukaryotic cell binding activity is associated with pentamers comprised of S2, S3, S4 and S5 polypeptides[12,13]. Non-toxic derivatives of PT have been constructed by substitution of key amino acids in the S1 subunit. Such mutations abolish ADP-ribosyltransferase activity but conserve other features of the protein, including holotoxin quaternary structure and eucaryotic cell binding[14]. One such PT derivative, PT-9K/129G, has been licensed as a component of a novel parenteral acellular pertussis vaccine[15].

Traditionally, toxins such as PT were inactivated by treatment with formaldehyde prior to incorporation in parenterally delivered acellular vaccines. However,

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recent reports suggest that if PT-9K/129G is treated with levels of formaldehyde which abolished eucaryotic cell binding (as measured by haemagglutinating activity) and mitogenic activities[16], the antibodies induced showed different specificity and toxin neutralizing properties than antibodies raised against the native protein. Treatment of this protein with low levels of formaldehyde (which did not affect cell binding or mitogenic activity) did not affect the specificity of the antibody induced but stablized the protein for long-term storage. Further work has suggested that heavy treatment with formaldehyde may constrain in the proteolytic processing and presentation of the treated antigen by T-cells[17].

B. pertussis is, as already stated, a pathogen which exclusively colonizes the surface of the respiratory mucosae. At present, both the whole cell vaccine and acellular vaccine are parenterally delivered. This route of immunization induces strong immunity to disease but may not prevent infection. However, induction of local responses following mucosal immunization may prevent colonization of the organism[18]. In this study we have analysed the mucosal immunogenicity of native and formaldehyde treated toxin using a murine model. Purified native PT-9K/129G or PT-9K/129G treated with different concentrations of formaldehyde, was administered to mice i.n. and s.c. Local and systemic immune responses to the different forms of the toxin were assessed. Here we report that HFT markedly diminishes the mucosal but not systemic immunogenicity of PT-9K/129G, whereas LFT does not significantly affect the response to mucosal or systemic immunization.

MATERIALS AND METHODS

Preparation of PT-9K/129G and treatment with formaldehyde

PT-9K/129G was prepared as previously described[14]. Formaldehyde treatment was carried out with either 0.15% formaldehyde (light treatment, LFT) only or 5% formaldehyde plus 25 mg ml^{-1} lysine (heavy treatment, HFT) for 48 h at 37°C. The samples were then dialysed exhaustively against saline (0.9% NaCl) and protein content analysed[14]. The formalin treatment of both proteins was stronger than that normally used to stablize PT-9K/129G for vaccine production, in which the protein is treated with 0.03% formalin[16,19]. Haemagglutinating activities for untreated, LFT or HFT PT-9K/129G were 0.5 μg ml^{-1}, 10 μg ml^{-1} and 100 μg ml^{-1} respectively[16]. For immunizations, proteins were appropriately diluted in PBS.

Immunization of mice

Adult female (6–8-week-old) BALB/c mice (Charles River, Margate, Kent, UK) were used throughout. Mice were immunized subcutaneous (s.c.) in the nape of the neck with 100 μl of vaccine containing 10 μg of PT-9K/129G or its derivatives and 0.5% (w/v) alhydrogel. Proteins were adsorbed to alhydrogel overnight at 4°C.

Intranasally immunized mice were lightly anaesthetized with halothane and 10 μg of protein applied to the nares (15 μl per nostril) by micropipette. Mice were immunized s.c. and i.n. on day 1 and all mice were boosted on days 23 and 37. Serum samples were taken on days 0, 22 and 36 and mice were terminally exsanguinated on day 51. Lung and nasal washings were performed on day 51. Nasal washes were obtained post mortem by cannulation of the nasopharyngeal orifice at the rear of the soft palate with a fine tipped pipette (Alpha Labs, Hants, UK) and lavage of the nasal cavity with 1 ml 0.1% bovine serum albumin in PBS (0.1% BSA). Lung washes were obtained similarly by cannulation of the trachea with a fine tipped pipette at the level of the thyroid and lungs were washed with 1.0 ml 0.1% BSA. Sera and lavages were stored at −40°C until analysed.

Measurement of serum antibody

Anti-toxin antibodies were measured by kinetic ELISA. Briefly, 96-well EIA/RIA plates (Costar, High Wycombe, Bucks, UK) were coated overnight at 4°C with 50 μl of homologous antigen at a concentration of 2.5 μg ml^{-1}. Plates were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-T; Sigma, Poole, UK) and blocked with 1% BSA for 2 h at 37°C. Plates were washed thrice and incubated with serial dilutions of serum or lavages for 2 h at 37°C. All samples and reagents were diluted in PBS-T plus 0.1% BSA. Total anti-toxin immunoglobulin was measured by incubation of the plates with rabbit anti-mouse horse radish peroxidase (HRP) conjugated antibody (Dako, High Wycome, UK) for 2 h at 37°C. IgA specific antibody was determined by incubation of the plates firstly, with α-chain specific biotin-conjugated goat anti-mouse immunoglobulin (Sigma) for 1 h at 37°C, and secondly with HRP-conjugated streptavidin (Dako) for 1 h at 37°C. Bound antibody was visualized by adding o-phenylenediamine substrate (0.04% substrate in citrate–phosphate buffer, pH 5 containing 0.01% (v/v) H_2O_2). The rate of colour deveolpment was monitored by absorbance at 450 nm (Ceres 900 ELISA reader, KinetiCalc II software, Bio-Tek, Winooski, VT, USA). The rate of substrate breakdown was plotted against dilutions and titres of total serum antibodies were determined relative to a positive serum, obtained from a pool of mice immunized s.c. with PT-9K/129G, which was given an arbitrary value of 1000.

Measurement of local IgA in nasal and lung lavage

Local IgA levels are expressed as a ratio of specific anti-toxin IgA detected in the lavage against the total amount of IgA measured in the same wash.

Specific anti-toxin IgA was detected as described above. Titres were expressed as the reciprocal of the highest dilution of sample which gave an OD value of 0.1 after subtraction of non-immunized control nasal and lung lavages.

Total IgA antibodies in nasal and lung lavage were measured by kinetic ELISA as described above, except that 96 well plates were coated overnight at 4°C with 50 μl of α-chain specific goat anti-mouse immunoglobulin (Sigma) at a concentration of 5 μg ml^{-1} in carbonate buffer. Plates were washed and blocked as described above before the addition of serial dilutions of lavages for 2 h at 37°C. Plates were washed and incubated with α-chain specific biotin conjugated goat

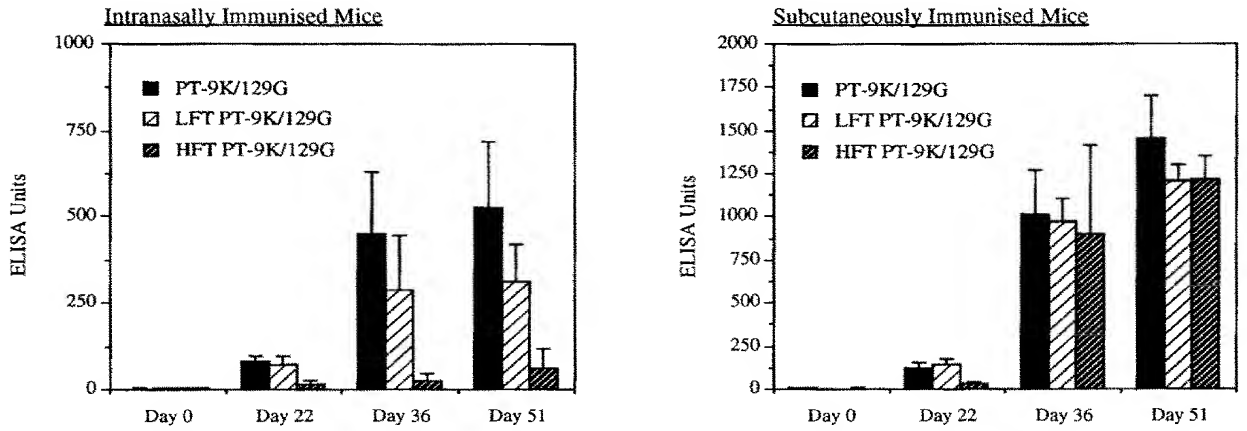

Figure 1 Total immunoglobulin responses in the sera of mice immunized i.n. (a) and s.c. (b) with untreated, LFT or HFT forms of PT-9K/129G. Mice were immunized on days 1, 22 and 36. Error bars represent the standard deviation of the mean of 4 i.n. immunized mice and 2 s.c. immunized mice

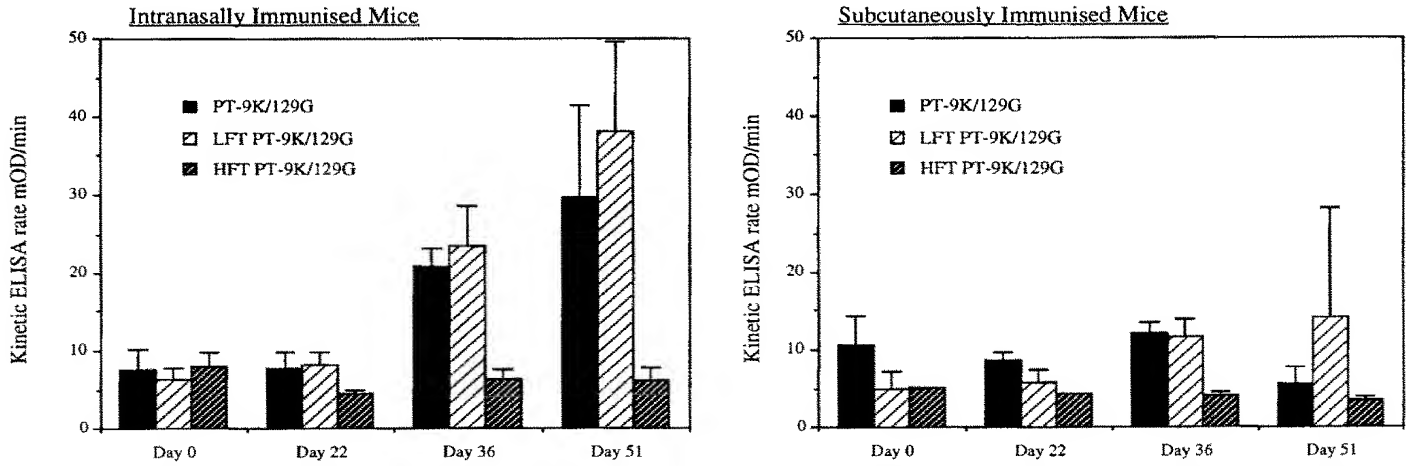

Figure 2 IgA responses in the sera of mice immunized i.n. (a) and s.c. (b) with untreated, LFT or HFT forms of PT-9K/129G. Mice were immunized on days 1, 22 and 37. Columns on this graph represent the average rate of change of substrate (mOD min^{-1}) over 5 min, in wells in which the sera was diluted 1:50

anti-mouse sera and the assay completed as described above. Concentration of IgA were calculated from a standard curve created using mouse myeloma IgA (ICN, Oxford, UK).

Results were calculated by dividing the titre of specific IgA by the total IgA (μg) in the sample.

Measurement of serum PT-neutralizing activity

This was measured by neutralization of PT-mediated Chinese Hamster Ovary (CHO) cell cytotoxicity as described previously[16] on individual sera collected on day 51 of this experiment.

RESULTS

Intranasal immunization with PT-9K/129G

Serum anti-PT responses. Mice immunized i.n. with untreated native PT-9K/129G developed anti-toxin antibodies in their serum, as did mice immunized i.n. with LFT PT-9K/129G (*Figure 1*). Small responses, which were observed following a single i.n. dose, were boosted in both groups following a second immunization. However, little change in serum responses were observed following a third i.n. dose. These responses were lower than those obtained following s.c. immunization but were still relatively high. In contrast mice immunized i.n. with HFT PT-9K/129G developed only a very poor serum response even after three i.n. immunizations. Statistically, these responses were significantly lower than those titres observed in mice immunized i.n. with PT-9K/129G or LFT PT-9K/129G ($P<0.005$) ($P<0.01$). In contrast, the responses induced by the different PT-9K/129G preparations, following s.c. immunization, were not significantly different.

In addition, only proteins administered i.n. elicited a small but measurable specific anti-PT IgA response in the serum (*Figure 2*). This response was also significantly affected by the formaldehyde pretreatment of the toxin. Both the untreated and LFT PT-9K/129G induced a small but measurable serum IgA response whilst HFT PT-9K/129G did not. The kinetics of the response paralleled the total antibody response with detectable antibodies observed after the second dose, however, these levels were boosted after a third immunization.

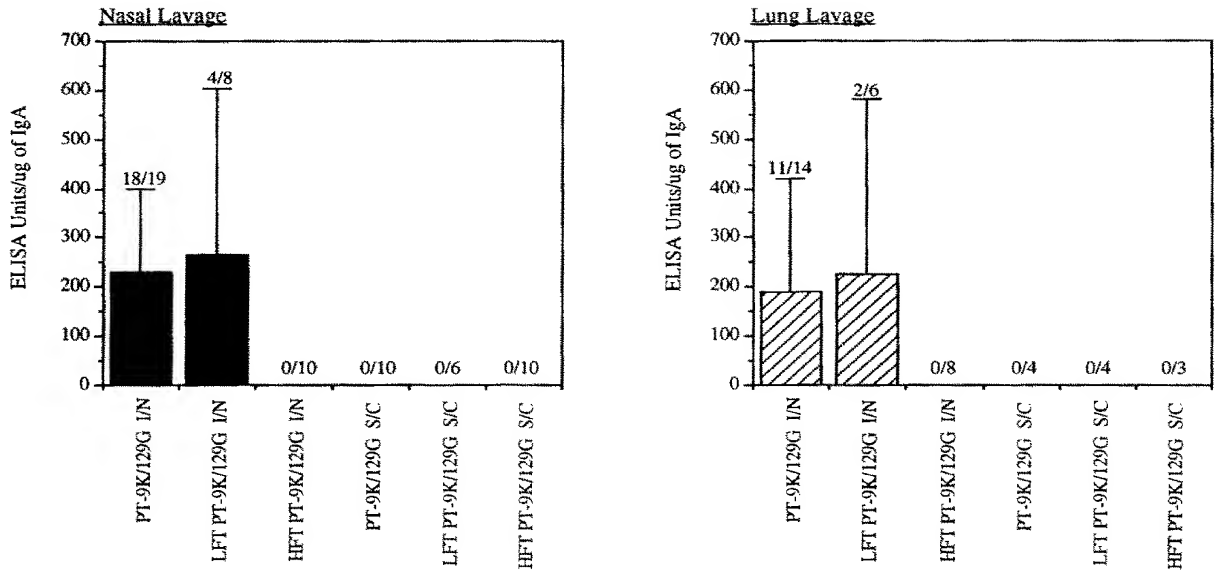

Figure 3 IgA responses in nasal (a) and lung lavage (b) of mice immunized i.n. and s.c. with the three different forms of the toxin. Responses are given as ELISA units per μg of IgA in the sample. Numbers above the columns represent the number of animals in which toxin specific antibodies were detected

Local anti-PT-9K/129G antibody responses

Local IgA responses were monitored in lavage samples taken from mice immunized with the three different versions of PT-9K/129G. Anti-PT-9K/129G IgA was not detected in the lung or nose washings of any of the mice immunized s.c. or following i.n. immunization with HFT PT-9K/129G (*Figure 3*). However, IgA anti-PT-9K/129G responses were detectable in some of the lung washings and in nearly all of the nasal washes from mice immunized i.n. with either, untreated or LFT PT-9K/129G. These responses were very variable, with some mice showing very good responses whilst others showed no response at all. However, when the overall responses from mice taken from several different experiments were considered, it was clear that only mice immunized with untreated or LFT showed specific anti-PT responses. These responses were relatively weak, especially when compared to anti-LT IgA responses induced by a non-ADP-ribosylating mutant of LT. The responses in these mice immunized with an equivalent dose of toxin were approximately five times higher (data not shown).

PT-NEUTRALIZING SERUM RESPONSE

The toxin-neutralizing activity of sera taken on day 51 was assessed in the CHO cell assay. Results from this assay are shown in *Table 1*. These indicate that all forms of PT-9K/129G induced a neutralizing antibody response following s.c. immunization. These results are in direct contrast to Nencioni *et al.*[16] who found a higher titre of toxin neutralizing antibody in guinea pigs immunized with the untreated form of the toxin and low levels of neutralizing activity in animals immunized with heavily treated toxin. However, the sera in these experiments was obtained from guinea pigs which were immunized intradermally and cannot be directly compared.

Interestingly, following i.n. immunization with untreated or LFT PT-9K/129G, low but specific neutralizing activity was detected in the sera. Although the levels of neutralizing antibodies were low the pattern of these results were consistent in different experiments. By contrast, sera obtained from mice immunized i.n. with HFT PT-9K/129G possessed a much lower neutralizing activity.

Table 1 Reciprocal titres of toxin neutralizing antibody in the serum of mice immunized i.n. or s.c. with untreated, lightly or heavily formaldehyde treated versions of PT-9K/129G. Sera of all mice immunized i.n. with untreated or lightly treated PT-9K/129G showed titres which varied from 1/40 to 1/160. In contrast, three of the four mice immunized with the heavily treated toxin showed no activity above background (1/10)

Route of immunization	PT-9K/129G pretreatment	Serum CHO neutralization titres (reciprocal)±S.D.
Intranasal	Untreated	100±69.3
	LFT	140±40
	HFT	27.5±35
Subcutaneous	Untreated	240±113
	LFT	640±0
	HFT	960±453

DISCUSSION

In this report we show that native recombinant PT-9K/129G, a non-toxic site-directed mutant of pertussis toxin, is able to act as a mucosal immunogen following intranasal administration of low doses of antigen to mice, eliciting both local IgA and serum antibodies. Mucosal immunogenicity was retained if PT-9K/129G was lightly treated with formaldehyde, but dramatically reduced if treated with formaldehyde in a manner which abolished the ability of the toxin to bind eucaryotic cells (as defined by haemagglutination activity). PT-9K/129G is therefore, one of the small group of pure proteins able to act as effective mucosal immunogens. Attempts to define common properties associated with effective

mucosal immunogens have been limited in the past. Aizupurua and Russell-Jones[20] classified proteins for their ability to act as mucosal immunogens following oral administration. They identified several effective mucosal immunogens, including pili from enteric bacteria, and concluded that a common feature of these proteins was that they were able to bind to eucaryotic cells. Indeed the immunogenicity of some pili was reduced if they were pre-treated with sugar molecules that blocked their receptor-binding sites. However, Elson and co-workers[21] found that a number of proteins with affinity for molecules on the surface of eucaryotic cells, including active pertussis toxin, were not good oral immunogens. Thus other factors in addition to cell binding must play a role in oral immunogenicity. Such factors could include the ability to resist degradation by proteases present at different mucosal surfaces and in different animal species.

The best characterized mucosal immunogens are CT, LT and their B subunits. These proteins are effective mucosal immunogens in a number of animal species when presented either orally or i.n.[6–10,22,23]. Early studies comparing native CT and B subunit or formaldehyde-treated toxoid, showed that effective gut mucosal immunogenicity was associated with the ability of the native toxins to bind to gangliosides at the surface of mucosal cells, as formaldehyde toxoided material was not an effective mucosal immunogen. In this study HFT PT-9K/129G did not greatly affect the parenteral immunogenicity of this protein. However, HFT abolished the mucosal immunogenicity of mucosally presented PT-9K/129G and this correlates with the abolition of eucaryotic cell binding *in vitro*. Our results taken together with those described by earlier workers provides strong evidence that cell-binding is an important factor in mucosal immunogenicity, possibly assisting the presentation of antigen to the immune cells of the mucosal epithelium or mucosal-associated lymphoid tissues. More convincing proof could be obtained using site-directed mutants of CT, LT or PT that are unable to bind to cells but are still able to fold into the conformation of the holotoxin. The use of such mutants might avoid the potential effects of the cross-linking activity known to be associated with formaldehyde. Such mutants have been described for all of these toxins[14,24] and such studies are being undertaken.

PT-9K/129G does not appear to be as potent an i.n. immunogen as CT or CT-B. When these antigens are administered to BALB/c mice using identical protocols (Douce and Cropley, unpublished results), CT and CT-B are consistently more effective mucosal immunogens, inducing higher systemic and local antibody titres than PT-9K/129G. However, as the CT-B used in these experiments was provided from a commercial source and was possibly contaiminated with a small but significant amount of active A subunit, it is possible that differences are due to the effect of ADP-ribosylation. In addition, the major histocompatibility complex (MHC) background of mice greatly affects the immunogenicity of CT, CT-B and LT-B[25]. Therefore, the immunogenicity of PT may be enhanced in mice with a different MHC class restriction.

As infection of *B. pertussis* is restricted to the respiratory tract, it is likely that the local mucosal immune system plays a role in immunity to whooping cough. Epidemiological evidence suggests that the current parenterally administered whole cell vaccine prevents disease but not infection. This is because this mode of immunization does not effectively elicit a local antibody response. It is possible that vaccines, that prevent infection and disease, could be developed by the direct application of antigens to the mucosal surface of the respiratory tract. One approach to development of mucosal vaccines would be the selection of *B. pertussis* antigens which act as effective mucosal immunogens. Pertussis toxin and other *B. pertussis* antigens such as FHA and pertactin, are likely to be important components of future acellular whooping cough vaccines. Currently several parenteral acellular vaccines containing these antigens as key components are in development or clinical trials[11,15]. In these vaccine preparations the antigens are inactivated chemically and delivered parenterally. All three antigens (FHA, pertactin and PT) have been shown independently to be effective mucosal immunogens in murine models (Roberts, unpublished results, and Refs [18,26]). Pertactin and FHA are non-toxic and do not require pre-treatment with formaldehyde before inclusion in the vaccine. However, they are often treated with formaldehyde or related chemicals to inactivate PT, if they are all derived from virulent *B. pertussis* strains. Our results indicate that the levels of formaldehyde nominally used to toxoid PT would render PT useless as a mucosal immunogen. Genetic detoxification overcomes this problem and our studies show, in mice at least, that low levels of formaldehyde, required to stabilize the molecule, did not affect the i.n. immunogenicity of this protein. Preliminary data suggests that the antigens are still highly immunogenic when administered i.n. as a mixture and afford a degree of protection in challenge studies (Cropley, unpublished observation). This approach is certainly worth investigating in the future.

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